

University of Groningen

Activation, apoptosis and clearance of neutrophils in Wegener's granulomatosis

Rossum, Aart Pieter van

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2005

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Rossum, A. P. V. (2005). *Activation, apoptosis and clearance of neutrophils in Wegener's granulomatosis*. s.n.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Chapter 10

Summary, Discussion and Perspectives

André P. van Rossum

SUMMARY

In this thesis the role of neutrophils in WG during the process of activation, apoptosis and clearance was investigated. In **part I** of this thesis, the pathogenic role of neutrophils in WG was explored, in particular in relation to membrane proteinase 3 expression. In **part II**, studies were conducted to explore the hypothesis that accumulation of unscavenged apoptotic or necrotic neutrophils in leukocytoclastic tissue, often seen in WG, can be attributed to local overexpression of the prototypic tissue pentraxin PTX3 in these patients.

In **chapter 2**, an overview on the central role of neutrophils in WG is given. In WG, neutrophils are supposed to play this central role due to the presence of circulating ANCA (antineutrophil cytoplasmic autoantibodies) that can bind to pre-activated (primed) neutrophils expressing the target autoantigens of ANCA. Binding results in further activation including release of lytic enzymes and reactive oxygen species (ROS), which potentially harm (activated) endothelium. Indeed, neutrophils are present at sites of injury, as can be demonstrated in renal biopsies, and numbers of activated neutrophils correlate with renal damage. In addition, we reviewed literature supporting the broader role of neutrophils in the pathophysiology of ANCA-associated vasculitis. Following activation, neutrophils become apoptotic. In the apoptotic state, the target autoantigens PR3 and MPO are upregulated and expressed on the neutrophil membrane. ANCAs can bind and opsonize these apoptotic neutrophils. Opsonization of apoptotic neutrophils normally results in a pro-inflammatory clearance by phagocytosing cells as macrophages instead of anti-inflammatory clearance. Furthermore, opsonization of apoptotic neutrophils may lead to maturation of dendritic cells, favouring the presentation of the target antigens of ANCA by these dendritic cells to immuno-competent cells. As such, opsonization may lead to a perpetuation of inflammation and autoimmunity. Next to pro-inflammatory clearance of apoptotic neutrophils also disturbances in the clearance of these apoptotic cells in WG is observed. In WG, often an accumulation of unscavenged apoptotic or necrotic cells is seen in the skin. This is interesting, since normally dying cells are swiftly engulfed by phagocytosing cells. In line with this disturbed clearance, we reviewed supporting evidence for the hypothesis that a locally produced pentraxin PTX3 is a candidate factor in disturbing normal clearance of late apoptotic neutrophils.

As PR3- ANCA interact with the target autoantigen PR3 on the membrane of neutrophils, we discussed membrane proteinase 3 expression on neutrophils as a pathogenic factor on resting neutrophils in **chapter 3**. Membrane-bound proteinase 3 (mPR3) can be expressed on the total population or on a subset of neutrophils. The percentage of neutrophils expressing mPR3 ranges from 0 - 100% of the neutrophil population within individuals. Neutrophils that hardly express proteinase 3 on the membrane are designated as mPR3⁻ neutrophils, whereas neutrophils that express proteinase 3 on the membrane are called mPR3⁺ neutrophils. The percentage of neutrophils that express mPR3 is stable within an individual, and is supposed to be genetically determined. Clinically, the percentage of neutrophils expressing mPR3 in patients with WG is increased, and an increased expression of mPR3 is associated with an increased incidence and rate of relapse in patients with WG. Finally, we discussed

the hypothesis that the association between an increased risk of relapse and an increased expression of mPR3 on neutrophils can be explained by an increased susceptibility of mPR3⁺ neutrophils to become activated by PR3-ANCA in patients with WG.

As mentioned before, mPR3 expression can be present on the total population or on a subpopulation of neutrophils, ranging from 0% to 100%. This percentage is stable within an individual, and increased percentages have been observed in ANCA-associated vasculitis (AAV). However, conditions for assessment of mPR3 expression have not been analyzed. In **chapter 4** we described a study in which we have analyzed mPR3 expression on neutrophils before and after priming with TNF- α in order to assess whether standardized assessment of mPR3 expression requires priming. We found that in almost half of all bimodal donors analyzed, substantial mPR3 expression was only seen after priming with TNF- α . In these donors, a clear distinction between the mPR3⁻ and the mPR3⁺ subset could only accurately made after priming with TNF- α . Therefore, we concluded that standardized assessment of proteinase 3 on the membrane of neutrophils requires priming. Using primed neutrophils we assessed whether percentages of mPR3⁺ neutrophils are increased in patients with ANCA-associated vasculitis compared to disease- and healthy controls. We found that percentages of mPR3⁺ neutrophils after priming were increased in patients with PR3- and MPO-ANCA-associated vasculitis, and systemic lupus erythematosus, but not in patients with rheumatoid arthritis. Furthermore, we demonstrated that differential expression of mPR3 within bimodal donors did not result from differences in mobilization of secretory vesicles, in activation status or in TNF-Receptor responsiveness. Whereas a pathophysiological role for (increased) mPR3 expression has been strongly suggested in PR3-ANCA associated vasculitis; its role in other ANCA-associated vasculitides and SLE requires further studies.

In **chapter 5**, a most fundamental issue in the pathophysiology of WG was explored: that is, binding of PR3-ANCA to mPR3 expressing neutrophils in blood. Most of the studies reporting that PR3-ANCA can activate neutrophils via binding to membrane-bound PR3 have been conducted with isolated IgG from patients with positive titers of PR3-ANCA or artificially raised mouse- or rabbit-antibodies against human PR3 and isolated neutrophils. With this in mind, a German group reported that neutrophils from patients with positive PR3-ANCA titres *ex vivo* show binding of ANCA that had occurred *in vivo*. Furthermore, they observed that PR3-ANCA present in serum from patients do not bind to primed neutrophils, whereas isolated IgG from these sera and rabbit polyclonal antibodies to human PR3 did bind. Based upon these observations they stated that PR3-ANCA are low affinity antibodies, and, as such, are not capable of binding *in vivo*. Due to the challenging nature of this report, we undertook a study in which we explored binding of PR3-ANCA to mPR3-expressing neutrophils in more detail. We found that PR3-ANCA do bind to neutrophils that express proteinase 3 on the membrane under serum or plasma conditions. In addition, we demonstrated that binding of PR3-ANCA to mPR3 expressing neutrophils was concentration- dependent, independent of how PR3 was translocated to the membrane, and independent from Fc γ -receptor interactions. So, we reported that the hypothesis that binding of PR3-

ANCA to primed neutrophils, leading to neutrophil activation, is still valid, and is still the most attractive explanation for the contribution of PR3-ANCA to the pathogenesis of WG.

As described before, high expression of PR3 on the membrane of non-primed neutrophils is associated with an increased risk to relapse in patients with WG. To explain this phenomenon, we conducted a study (**chapter 6**) in which we related levels of mPR3 expression to neutrophil activation after stimulation with PR3-ANCA. Therefore, we used non-primed and primed neutrophils and measured early and late events in neutrophil activation after PR3-ANCA stimulation. Priming was done using low doses of the inflammatory cytokine TNF- α . We measured polymerisation of actin, a constituent of the cytoskeleton, as an early event in neutrophil activation, and oxidative burst by the dihydrorhodamine assay as a late event in neutrophil activation. We found that antibodies against PR3 could polymerize actin in neutrophils even without priming with TNF- α . In contrast, priming of neutrophils with TNF- α is a prerequisite for the oxidative burst to occur, which is probably due to effects of TNF- α on the assembly of the NADPH oxidase complex. Furthermore, we found a correlation between the level of mPR3 expression on non-primed neutrophils and the degree of actin polymerisation after stimulation with antibodies against PR3. However, no correlation could be observed between the level of mPR3 expression on primed neutrophils and levels of actin polymerization and oxidative burst. So, levels of mPR3 expression on non-primed neutrophils have direct consequences for their susceptibility to initial activation by PR3-ANCA. As such, these data may be relevant in view of the observed relation between patients who have a high expression of PR3 on the membrane of their non-primed neutrophils and their increased susceptibility for relapses.

As autoantigen expression on neutrophils is a prerequisite for activation by ANCA, we have studied signaling routes that are involved in translocation of PR3 to the membrane in **chapter 7**. Translocation of PR3 to the membrane is proposed to occur during priming with pro-inflammatory cytokines or during apoptosis. We demonstrate that, indeed, apoptosis results in translocation of PR3. However, translocation was only seen on a subset of neutrophils, and this subset equals the number that expressed mPR3 during priming with TNF- α in donors bimodal for mPR3 expression, that is having both mPR3⁻ and mPR3⁺ neutrophils. Next, we found that translocation of PR3 during apoptosis induced by combined treatment with TNF- α and cycloheximide, was dependent on p38MAPKinase and Phosphatidylinositol3-Kinase (PI3-K) activities. Blocking p38MAPK or PI3K activity resulted in an almost complete lack of mPR3 expression during apoptosis, whereas blocking did not affect apoptosis itself. Another way of inducing apoptosis in neutrophils is via UV-B induced irradiation. Expression of mPR3 during UV-B apoptosis was also dependent, although only partly, on p38MAPKinase activity, but not on PI3-Kinase activity. Finally, we demonstrated that translocation of PR3 during priming with TNF- α is dependent on p38MAPK as well, whereas not on PI3-K. So, translocation of PR3 to the membrane of neutrophils during priming and apoptosis is dependent on p38MAPKinase activity, and, dependent on the type of apoptosis induction, on PI3-Kinase activity. Therefore, pharmacological

inhibitors interfering with p38MAPK or PI3-K activity could be an interesting therapeutic strategy in the management of WG.

The second part of the thesis studied disturbances in the clearance of apoptotic neutrophils in vasculitis. In WG leukocytoclastic lesions are frequently found. These lesions are characterized by infiltration of neutrophils, endothelial swelling, and an accumulation of unscavenged apoptotic or necrotic neutrophils and fragmented nuclei around the vessel wall (leukocytoclasia). Accumulation of dying cells and the presence of nuclear debris in these lesions suggest that the removal of dying cells is incomplete. The factor(s) disturbing normal clearance of dying cells are yet unknown. In **chapter 8 and 9**, we hypothesize that the pentraxin PTX3 can be a candidate factor in inducing leukocytoclasia. PTX3 is locally produced at inflammatory sites, and structurally related to serum amyloid P (SAP) and C-reactive protein. In **chapter 8**, we demonstrate in an *in vitro* study that PTX3 inhibits the clearance of late apoptotic cells by macrophages. This contrasts with another pentraxin, SAP, which proved to facilitate clearance. Furthermore, we demonstrated that binding of apoptotic neutrophils to macrophages was partly affected by PTX3. In line with our proposed hypothesis that PTX3 induces leukocytoclasia in WG, we explored whether PTX3 is, indeed, present at sites of leukocytoclastic lesions (**chapter 9**). Therefore, we selected skin biopsies of 13 patients with leukocytoclastic vasculitis and stained these biopsies for the presence of PTX3. We found that PTX3 was abundantly upregulated in diseased skin compared to healthy controls. At these vasculitis sites, PTX3 was localized to endothelial cells or diffusely present throughout the affected tissue. Furthermore, we found a clear positive correlation between the amount of infiltrating cells and the level of PTX3 produced at these affected sites, as assessed by semi-quantitative analysis. Thus the inhibitory effects of PTX3 *in vitro* on the clearance of late apoptotic neutrophils, and the abundant presence of PTX3 *in vivo* at sites of leukocytoclastic lesions, strengthen the hypothesis that PTX3 is a candidate factor in inducing leukocytoclasia in patients with WG.

DISCUSSION AND PERSPECTIVES

Membrane proteinase 3 expression as a risk factor

The first question we addressed is whether proteinase 3 expression on the membrane of neutrophils is a risk factor for disease induction or disease expression in patients with WG. To address this question, we have first reviewed relevant literature on this issue. All studies uniformly demonstrate an increased percentage of neutrophils that express mPR3 in patients with WG¹⁻⁴. In these studies, non-primed neutrophils were analyzed for mPR3 expression. However, as has been shown in chapter 4, TNF- α -primed neutrophils are needed to accurately assess the maximal percentage of neutrophils able to express proteinase 3. mPR3 expression on non-primed neutrophils *in vitro* is probably due to stress events during isolation procedures, or even *in vivo* activation during active disease. Therefore, studies analyzing proteinase 3 expression on non-primed neutrophils can be biased by uncontrollable isolation procedures or differences in disease activity. It has been argued that these former studies are measuring constitutive expression of mPR3 on non-primed neutrophils, often regarded

as resting neutrophils. However, there are a number of arguments that refute the argument of constitutive mPR3 expression on resting neutrophils. Firstly, in whole blood techniques, mPR3 expression is only seen after stimulation or incubation. Secondly, we demonstrated in chapter 7 that even in evidently bimodal donors, almost all mPR3 expression on the mPR3⁺ subset disappears during culture. Thirdly, constitutive expression of mPR3 potentially leads to severe neutropenia, as PR3-ANCA would bind to constitutively expressed PR3 on resting neutrophils resulting in opsonization of these neutrophils. Normally, opsonized neutrophils are removed from the circulation by FcR-bearing cells. However, severe neutropenia is not observed in WG. Hence, constitutive expression of mPR3 on resting neutrophils seems unlikely. Therefore, we analyzed mPR3 expression on neutrophils that were primed with TNF- α . We showed that, within individuals, there is a constitutive (sub) population of neutrophils that is able to respond to pro-inflammatory stimuli as TNF- α with translocation of PR3 to the membrane, independent of differential mobilization of vesicles or responsiveness to TNF- α . Furthermore, we demonstrated that after priming with TNF- α , the (sub) population of neutrophils able to express mPR3 is increased in PR3-ANCA associated vasculitis, but also in MPO-ANCA-associated vasculitis and SLE. So, yes, percentages of neutrophils that are constitutively able to express proteinase 3 on their membrane are a risk factor for disease in patients with WG.

Membrane proteinase 3 expression and functional consequences

Membrane proteinase 3 expression is a risk factor in WG. How can this be explained? The most common hypothesis is that increased numbers of mPR3⁺ neutrophils or increased levels of mPR3 expression on the mPR3⁺ (sub) population predispose for susceptibility for PR3-ANCA-induced neutrophil activation. A prerequisite in this hypothesis is that binding occurs of PR3-ANCA to mPR3 expressing neutrophils *in vivo*. As this prerequisite was challenged by a German report by Abdel Salam et al ⁵, we explored binding of PR3-ANCA to mPR3 expressing neutrophils. We found, in contrast to the report from the German group, *in vitro* data fully compatible with binding of PR3-ANCA to neutrophils (chapter 5). We conducted binding experiments of PR3-ANCA to mPR3 expressing neutrophils under serum or plasma conditions, thereby trying to reach physiological conditions. Nevertheless, definite proof on binding of PR3-ANCA to mPR3 expressing neutrophils *in vivo* is hard to get. As even during active disease, *in vivo* primed neutrophils, and thus expressing mPR3 and prone to bind PR3-ANCA are likely to become attached to locally activated endothelium. As such, ANCA-bound neutrophils most probably do not circulate, and cannot be detected in peripheral blood. Thus, *ex vivo* peripheral neutrophils will even during active disease, most probably, not express mPR3, and therefore will not have bound PR3-ANCA. Another strategy would be to search for neutrophils that have bound ANCA within the affected tissue. However, due to internalization a high turnover of bound ANCA can be expected under pathophysiological conditions. As such, it is very hard to proof binding of PR3-ANCA to primed neutrophils even in tissue affected with vasculitis. Nevertheless, the hypothesis that PR3-ANCA bind and activate primed neutrophils is still the most attractive explanation for the contribution of PR3-ANCA to the pathogenesis of Wegener's granulomatosis.

The third question we addressed is whether increased levels of mPR3 on neutrophils associates with increased neutrophil activation after stimulation with anti-PR3 antibodies. This question is of particular interest in view of clinical data showing that increased levels of mPR3 expression on neutrophils are associated with an increased risk for relapses in WG. We demonstrate in chapter 6 that the level of mPR3 expression on non-primed neutrophils correlates with the degree of F-actin polymerization as an early activation marker after stimulation with anti-PR3 antibodies. Therefore, we may conclude that, indeed, increased levels of mPR3 expression on neutrophils do have consequences for their susceptibility to the initial activation step by anti-PR3 antibodies. This is further strengthened by a report of Schreiber et al ⁶ which demonstrated that mPR3⁺ neutrophils, after being separated from the mPR3⁻ neutrophils in bimodal mPR3 donors, show higher degrees of oxidative burst and degranulation than mPR3⁻ neutrophils after stimulation with PR3-ANCA. Furthermore, increasing the percentage of mPR3⁺ neutrophils compared to mPR3⁻ neutrophils in the responder population resulted in a gradual increase of the oxidative burst as induced by PR3-ANCA.

In conclusion, functional expression of membrane proteinase 3 at the cellular level is a causal explanation for membrane proteinase 3 expression being a risk factor for disease expression in WG.

Autoantigen expression as a therapeutic target

Expression of the autoantigen proteinase 3 (PR3) on the membrane is a prerequisite for activation of neutrophils by PR3-ANCA. As autoantigen expression plays such a dominant role in the pathophysiology of ANCA-associated vasculitis, we searched for regulatory proteins involved in the translocation of proteinase 3 to the membrane in order to reveal possible new therapeutic targets in the treatment of ANCA-associated vasculitis (chapter 7). Proteinase 3 can be translocated to the membrane during the process of priming or apoptosis. In vasculitis, increased numbers of primed neutrophils are found ⁷. Furthermore, these neutrophils undergo apoptosis more readily in conjunction with increased expression of autoantigens. We found that pharmacological inhibitors interfering with p38 MAPK and PI3-K activity can block the induced expression of mPR3 during TNF- α -accelerated apoptosis, while not affecting apoptosis itself. Furthermore, mPR3 induced expression during TNF- α priming was dependent on p38MAPK activation and could be blocked by pharmacological inhibitors interfering with p38MAPK activity as well. As autoantigen expression plays such a dominant role in the pathophysiology of ANCA-associated vasculitis, we should further search for regulatory molecules that control mPR3 expression, and for pharmacological inhibitors of these regulators in order to add new effective therapeutic strategies to the treatment of ANCA-associated vasculitis. In concordance, current immunosuppressive drugs used in treatment strategies of patients with WG should be analyzed for their potential to interfere with mPR3 expression on neutrophils as well.

Leukocytoclasia explained by overexpression of PTX3?

In leukocytoclastic vasculitis an accumulation of unscavenged apoptotic or necrotic neutrophils and fragmented nuclei around the vessel wall (leukocytoclasia) is observed. In this thesis, supporting data are presented for the hypothesis that the

pentraxin PTX3 is a candidate factor in inducing leukocytoclasia. We demonstrate that *in vitro* PTX3 inhibits clearance of late apoptotic cells by macrophages. Furthermore, we found that PTX3 was abundantly upregulated in diseased skin compared to healthy controls. PTX3 was localized to endothelial cells or diffusely present throughout the affected tissue and the level of PTX3 produced at these affected sites correlated with the amount of infiltrating cells. As soluble PTX3 inhibits complement activation by blocking interactions of C1q with immunoglobulins⁸, this lack of complement activation could explain the disturbed clearance in the presence of PTX3. However, also other types of apoptotic cells should be inhibited in their uptake by phagocytosing cells as well, as complement activation generally facilitate uptake. Another explanation could be that direct “coating” of late apoptotic neutrophils with PTX3 is in a direct way responsible for decreased uptake by frustrating the recognition and internalization of these neutrophils by macrophages by yet unknown mechanisms. Therefore, mechanistic proof for the mode of action is awaiting. Other evidence supporting the role of PTX3 in leukocytoclasia should originate from experimental animal models, e.g. by showing that intra-dermally delivered PTX3 at high concentrations, indeed, results locally at sites of neutrophilic infiltrates in the accumulation of unscavenged dying neutrophils.

REFERENCES

- 1 Csernok E, Schmitt WH, Ernst M, Bainton DF, Gross WL. Membrane surface proteinase 3 expression and intracytoplasmic immunoglobulin on neutrophils from patients with ANCA-associated vasculitides. *Adv.Exp.Med.Biol.* 1993; 336: 45-50.
- 2 Rarok AA, Stegeman CA, Limburg PC, Kallenberg CG. Neutrophil membrane expression of proteinase 3 (PR3) is related to relapse in PR3-ANCA-associated vasculitis. *J.Am.Soc.Nephrol.* 2002; 13: 2232-8.
- 3 Schreiber A, Busjahn A, Luft FC, Kettritz R. Membrane expression of proteinase 3 is genetically determined. *J.Am.Soc.Nephrol.* 2003; 14: 68-75.
- 4 Witko-Sarsat V, Lesavre P, Lopez S et al. A large subset of neutrophils expressing membrane proteinase 3 is a risk factor for vasculitis and rheumatoid arthritis. *J.Am.Soc.Nephrol.* 1999; 10: 1224-33.
- 5 Abdel-Salam B, Iking-Konert C, Schneider M, Andrassy K, Hansch GM. Autoantibodies to neutrophil cytoplasmic antigens (ANCA) do not bind to polymorphonuclear neutrophils in blood. *Kidney Int.* 2004; 66: 1009-17.
- 6 Schreiber A, Luft FC, Kettritz R. Membrane proteinase 3 expression and ANCA-induced neutrophil activation. *Kidney Int.* 2004; 65: 2172-83.
- 7 Harper L, Cockwell P, Adu D, Savage CO. Neutrophil priming and apoptosis in anti-neutrophil cytoplasmic autoantibody-associated vasculitis. *Kidney Int.* 2001; 59: 1729-38.
- 8 Nauta AJ, Bottazzi B, Mantovani A et al. Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. *Eur.J.Immunol.* 2003; 33: 465-73.